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{Exhibit 64}

Szostak et al., "Hybridization with Synthetic Oligonucleotides," Methods in Enzymol. 68: 419-429 (1979)

partially purified probe. Small quantities of supercoiled hybrid plasmid DNA are required, since the efficiency of transformation of competent *E. coli* strain RR101 with supercoiled DNA is 500–2000 transformants per nanogram of DNA.

The glyceraldehyde-3-phosphate dehydrogenase structural gene reported here has been extensively characterized by restriction endonuclease and transcriptional mapping analysis. A portion of the nucleotide sequence of the isolated structural gene has been determined and shown to encode a portion of the known amino acid sequence of yeast glyceraldehyde-3-phosphate dehydrogenase. These results will be reported elsewhere.

[29] Hybridization with Synthetic Oligonucleotides

By J. W. SZOSTAK, J. I. STILES, B.-K. TYE, P. CHIU, F. SHERMAN, and RAY WU

Recent advances in chemical[1] and enzymatic[2] synthesis of oligonucleotides have greatly increased the availability of these compounds. Oligonucleotides 10 to 20 bases long are potentially useful as hybridization probes for the detection of unique genes in Southern blot filter hybridization experiments[3] and for the screening of colony or bacteriophage banks for particular sequences. The amino acid sequence of many interesting proteins has been determined. From this information it is possible to deduce a partial nucleotide sequence for the corresponding mRNA or gene. The degeneracy of the genetic code results in ambiguity at the second base of some codons and at the third base of most codons. This effect can be minimized by selecting a region of the protein sequence consisting predominantly of unique codons (Met and Trp) and the other least ambiguous codons (Asp, Asn, Cys, His, Phe, Tyr, Glu, Gln, and Lys). In these cases, the uncertainty is between A and G or T and C, and the effect of possible mismatches is minimized by selecting G for A or G ambiguity and T for T or C ambiguity in the oligonucleotide.[4] This results in either correct base pairing or a G = T mismatch. This type of mismatch is expected to cause less destabilization of the helix than any other mismatch.[5]

In this chapter we describe procedures for the use of synthetic oligo-

[1] R. Wu, C. P. Bahl, and S. A. Narang, *Prog. Nucleic Acid Res. Mol. Biol.* **21**, 101 (1978).
[2] S. Gillam, R. Jahnke, and M. Smith, *J. Biol. Chem.* **253**, 2532 (1978).
[3] E. Southern, *J. Mol. Biol.* **98**, 503 (1975).
[4] R. Wu, *Nature (London), New Biol.* **236**, 198 (1972).
[5] O. C. Uhlenbeck, F. H. Martin, and P. Doty, *J. Mol. Biol.* **57**, 217 (1971).

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nucleotides for Southern blot experiments and gene bank screening, and demonstrate the effect of various mismatches on the efficiency of hybridization.

Sensitivity Versus Specificity

To use synthetic oligonucleotide probes for hybridization they must first be end-labeled and then annealed with single-stranded DNA bound to a nitrocellulose filter. The temperature should be 15°–20° below the estimated T_m of the hybrid; in practice the conditions of the hybridization reaction must be carefully optimized in order to achieve high sensitivity and specificity. The specificity of the probe is determined by its length (and therefore the number of times its complementary sequence occurs in the DNA being probed), and by the stringency of the reaction conditions. If the conditions are insufficiently stringent, the probe will hybridize with many closely related sequences. However, the efficiency of the hybridization reaction declines as the reaction conditions are made more stringent. A balance must therefore be found between the opposing requirements of sensitivity and specificity.

Hybridization with a 12-nucleotide-long fragment (12-mer), with one mismatch, is sufficiently sensitive for the detection of correct binding to a restriction digest of λ DNA. We have tested the effects of several different mismatches and find that errors near the middle of the sequence are more critical than errors near either end.

An oligonucleotide 13–15 nucleotides in length is sufficient for the detection of a unique gene in total yeast DNA by Southern blot analysis,[3] and a 15-mer can be used in the screening of a yeast DNA bank cloned in a λ vector. Conditions must be optimized and the specificity determined by Southern blot experiments before plaque hybridization is performed. However, analysis of mammalian DNA by Southern blots may not be possible because of the greater complexity of this DNA, although plaques could still be detected. The use of single-stranded DNA phages such as certain M13 derivatives as cloning vectors may considerably enhance the usefulness of oligonucleotide hybridization in gene bank screening because of the greater sensitivity attainable with these vectors.

Materials

Oligonucleotides: d(A-G-C-A-C-C-T-T-T-C-T-T-A-G-C), complementary to bases 24–39 of the yeast iso-1-cytochrome *c* mRNA, was synthesized in our laboratory[6] by the phosphotriester method. d(G-A-G-C-

[6] J. W. Szostak, J. I. Stiles, C. P. Bahl, and R. Wu, *Nature (London)* **265**, 61 (1977).

G-G-A-T-A-A-C-A-A-T-T) and d(C-C-G-C-T-C-A-C-A-A-T-T, complementary to the *Escherichia coli lac* operator, were a gift from S. A. Narang.

5′-End-labeling was carried out with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear) at a specific activity of 1000–3000 Ci/mmol.[7]

DNA: Yeast DNA was prepared as described[8] from the following strains: D311-3A (a *his1 trp2 lys2*), used as a standard *CYC1* (wild type) strain; B-955 (a *CYC1-91-A his1 trp2 lys2*) which has CAA in place of GAA[9] and thus lacks the *Eco*RI site at bases 3–9; B-2185 (a *CYC1-183-AD his1 trp2 lys2*) which has a deletion encompassing amino acids 8–12[10], the binding site of the 15-mer; ϕ80 *dlac* O^c mutants RV1, RV10, RV17, RV51, RV116, and RV120 which were a gift from J. R. Sadler.[11]

Restriction enzymes: Purchased from New England Biolabs.

Hybridization: DNA fragments were transferred to nitrocellulose paper (Schleicher and Schuell) by the blotting procedure of Southern[3] or the plaque transfer procedure of Benton and Davis.[12] All hybridizations were carried out in 2× SSC, with 0.2% polyvinylpyrrolidone, Ficoll, and bovine albumin (Sigma) in sealed plastic bags.

Hybridization of a 12-mer to the λ Endolysin Gene

The amino acid sequence of the λ endolysin gene is known[13] and contains a region with low-nucleotide-sequence ambiguity. We[14] synthesized a 12-mer complementary to this sequence, with four possible ambiguities of the G = T type. Hybridization to exonuclease III-digested λ DNA was observed, and primer extension and DNA sequencing work showed that hybridization with the correct site had occurred. It was not possible, however, to determine how many mismatched base pairs, if any, were actually present.

[7] A. Maxam, and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560 (1977).
[8] D. R. Cryer, R. Eccleshall, and J. Marmur, *Methods Cell Biol.* **12**, 39 (1975).
[9] F. Sherman and J. W. Stewart, *in* "The Biochemistry of Gene Expression in Higher Organisms" (J. K. Pollak and J. W. Lee, eds.), p. 56. Australian and New Zealand Book Co., LTD, Sydney, Australia, 1973.
[10] J. W. Stewart and F. Sherman, *in* "Molecular and Environmental Aspects of Mutagenesis" (L. Pradash *et al.*, eds.), p. 102. Thomas, Springfield, Illinois, 1974.
[11] A. Jobe, J. R. Sadler, and S. Bourgeois, *J. Mol. Biol.* **85**, 231 (1974).
[12] W. D. Benton and R. W. Davis, *Science* **196**, 205 (1977).
[13] M. Imada and A. Tsugita, *Nature (London), New Biol.* **233**, 230 (1971).
[14] R. Wu, C. D. Tu, and R. Padmanabhan, *Biochem. Biophys. Res. Commun.* **55**, 1092 (1973).

Hybridization of Oligonucleotide Probes to the *Escherichia coli lac* Operator

The effect of type and position of mismatch on oligonucleotide hybridization was tested. A-C, C-T, G-T, and G-A mismatches near the middle or ends of the complementary sequence were studied. The mismatches occurred when hybridizing DNA from a series of ϕ80 d*lac* O^c mutants to synthetic single-stranded *lac* operator probes carrying the unmutated sequence. Hybridizations were carried out at different temperatures to determine the stability of the hybrids. The position and base change of each mutation[15] are shown along with the sequence of the probes.

1. A synthetic 15-mer was hybridized with two mutants:

```
5'  G-A-G-C-G-G-A-T-A-A-C-A-A-T-T  3'     15-mer probe
3'—C-T-C-G-C-C-T-A-T-T-G-T-T-A-A—5'     lac operator sequence
    ↓ ↓
    A C                                  Single base change
 RV10 17                                 Mutant strain
```

DNAs, isolated from the ϕ80 d*lac* O^c mutants and from the wild type RV80, were digested with restriction enzyme *Eco*RI and analyzed after agarose gel electrophoresis by Southern blotting.[3]

Hybridization occurred specifically to one fragment size. At 31°, RV17 DNA hybridized with the 15-mer as well as RV80, but RV10 was significantly weaker. This means that a G-A mismatch on the end of the complementary sequence is more destabilizing than an A-C mismatch in the penultimate position. A mismatch on the end would presumably be less destabilizing than one further in. This advantage is probably nulled by the destabilization of the neighboring base pair by the purine to purine mismatch.[16] Thus, the relative stabilities of these hybrids is determined by mismatch type rather than position.

2. A synthetic 12-mer was hybridized to *Hin*dIII digested DNA from several of the ϕ80 d*lac* O^c mutants, shown below:

```
     1  3  5  7  9  11
5'   C-C-G-C-T-C-A-C-A-A-T-T  3'     12-mer probe
3'—G-G-C-G-A-G-T-G-T-T-A-A—5'     lac operator sequence
     ↓ ↓ ↓ ↓ ↓  ↓
     A T T G T  A                    Single base change
 RV120  51  10  1                    Mutant strain
      116  17
```

DNA from ϕ80 (lacking the d*lac*) as well as the wild type RV80 were

[15] W. Gilbert, J. Gralla, J. Majors, and A. Maxam, *in* "Protein-Ligand Interactions" (H. Sund and G. Blauer, eds.), p. 193, de Gruyter, Berlin, 1975.

[16] J. B. Dodgson and R. D. Wells, *Biochem.* **16**, 2367 (1977).

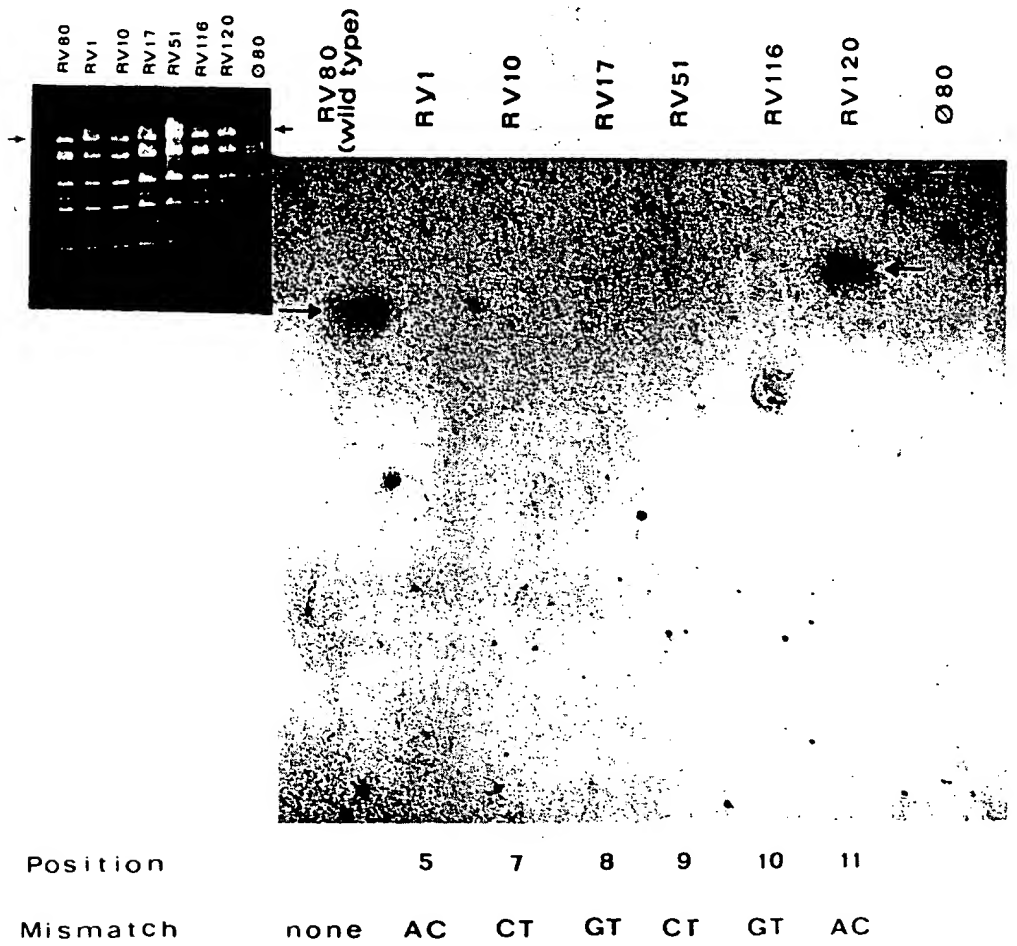

FIG. 1. Hybridization at 30° of a synthetic 12-mer probe to the *E. coli lac* operator region in ϕ80 d*lac* DNA. Insert shows the ethidium bromide stained gel of *Hin*dIII digested ϕ80 d*lac* DNA. The arrows in the insert indicate the *Hin*dIII fragment containing the *lac* operator sequence, and the two arrows in the figure show the location of the hybridized bands in RV80 and RV120. The filter was prehybridized in 5 ml of 10X Denhardt's solution made with 2XSSC for 14 hr at 65°. ^{32}P-labeled probe was added to a concentration of 3×10^5 cpm/ml. Hybridization was carried out at 30° for 13 hr. The hybridized filter was washed at 23° in 2XSSC for 2 hr, with 4 changes of buffer, before using to expose x-ray film.

included as controls. The results at 30° are shown in Fig. 1. The synthetic 12-mer probe hybridized to RV120 DNA as strongly as to RV80 DNA. At a more stringent temperature (32°) this signal was weaker than RV80, and at 37°, only the wild type hybridized. The mismatch with RV120 DNA is C-A which is presumably less stable than a G-T mismatch, such as found with RV116 and RV17. Hybridizations with the 15-mer, however, indicated that an A-C mismatch is relatively stable at 31°. Thus, the determining factor for stable hybridization to the 12-mer is position rather than the nature of the mismatched base pair. The importance of position is especially striking when comparing RV116 and 120, in which the mismatches are G-T at position 3 and C-A at position 2, respectively. One

explanation is that in an oligonucleotide as short as the 12-mer probe, all the nucleotides on the short side of the mismatch are unpaired. The stability of hydridization then depends simply on a sufficient number of perfect base pairings. The number of base pairs before the mismatch minus one (due to the destabilizing effect of the mismatch[16]) can be termed the effective probe size. With RV116 the effective probe size is 8 and with RV120 it is 9. In the conditions of the experiment, a minimum effective probe size of 9 (with 4 G-C pairs) seems to be required for stable hybridization. This finding is in general agreement with other published results.[4]

Hybridization of Synthetic Probes to the *CYC1* Gene of Yeast

The sequence of the first 44 nucleotides of the yeast *CYC1* gene was determined by Stewart and Sherman[10] by amino acid sequence analysis of frameshift mutants. A 15-mer complementary to part of this sequence was synthesized for use as a hybridization probe by Szostak *et al.*[6] This 15-mer has been used in two ways to detect the *CYC1* gene in Southern blot experiments. First, the 15-mer was used to make cDNA complementary to the *CYC1* mRNA as previously described.[6] The labeled cDNA is a highly specific and sensitive probe, but it is difficult to prepare and is available in only small amounts. Second, end-labeled 15-mer was used directly as a hybridization probe. While this probe is easily prepared and is sufficiently specific, the efficiency of the hybridization is low and a large excess of labeled probe must be used.

Yeast DNA from a wild-type strain has an *Eco*RI site at bases 3–9 of the CYC1 gene (amino acids 2–3 of the iso-1-cytochrome *c*), whereas DNA from a strain carrying the *CYC1-91-A* mutation does not have this *Eco*R1 site. Since the strains are isogenic, we expect that the *CYC1* *Eco*R1 fragment will be larger in the mutant than in the wild type, while all other *Eco*R1 fragments will be of the same size. DNA from the two strains was digested with excess *Eco*R1, electrophoresed on a 1% agarose gel, and transferred to nitrocellulose paper. The filter was annealed with *CYC1* cDNA (50–150 bases long) at 65°, washed, and autoradiographed (Fig. 2). Hybridization was observed with DNA fragments of 4.45 Md from the wild-type strain, and 6.2 Md in the mutant. The size shift conclusively identified these fragments as carrying the *CYC1* gene. The 6.2-Md fragment was the fusion product of the 4.45- and 1.75-Md fragments from the wild-type strain.

In a separate experiment, hybridization of end-labeled 15-mer alone was observed with the 1.75-Md fragment. This fragment did not appear with DNA from a strain carrying the *CYC1-183-Ad* deletion which exactly

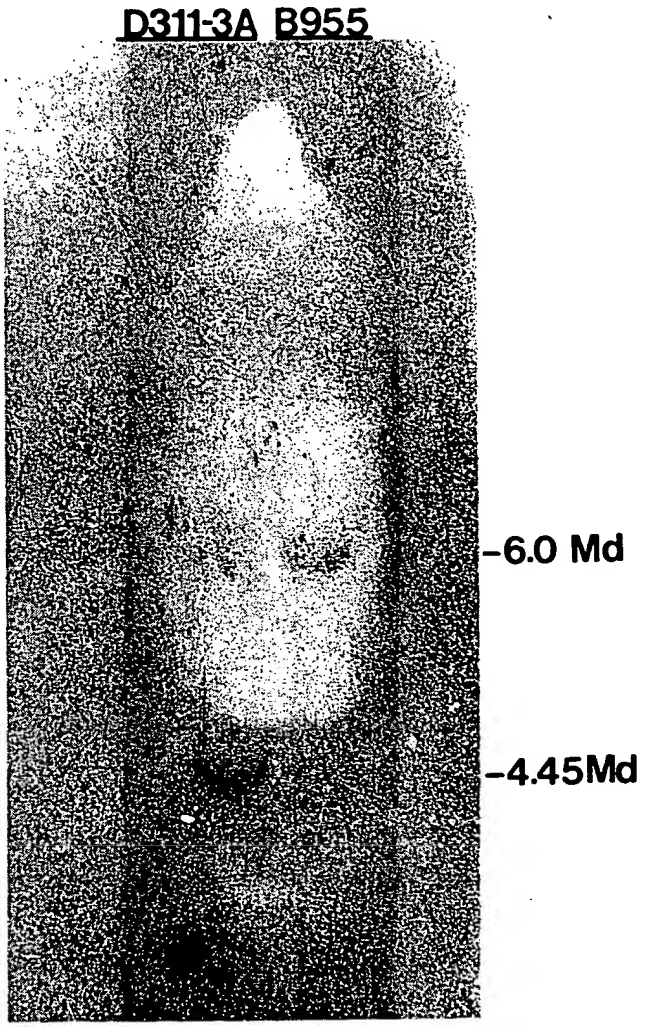

FIG. 2. Hybridization of a polymerase-extended 15-mer (*CYC1* cDNA) with yeast DNA from the wild type (D311-3A) and from a mutant lacking the *Eco*RI site (B-955). Loss of the *Eco*RI site results in the fusion of two adjacent fragments to give the one larger fragment (6.2 Md) seen in the mutant.

covered the 15-mer binding site. The 1.75-Md fragment must have been on the 3′-end of the 6.2-Md fragment and was the fragment carrying the structural gene for iso-1-cytochrome *c*.

Hybridization of labeled 15-mer to *Eco*R1-, *Bam*HI-, and *Eco*R1 plus *Bam*HI-digested wild-type yeast DNA is illustrated in Fig. 3. In each case there is a major and a minor band. The major band in the *Eco*RI digest is the 1.75-Md fragment carrying the *CYC1* gene. The minor band is probably due to a DNA fragment carrying a closely related sequence that also binds the 15-mer.

Hybridization was repeated at several temperatures between 25° and 50°. The optimum for sensitivity and specificity was in the 42°–45° range.

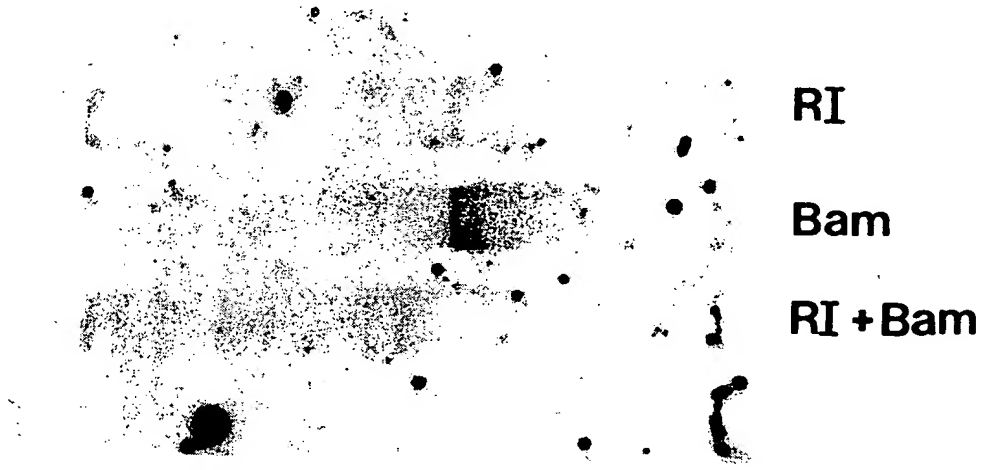

FIG. 3. Hybridization of a labeled 15-mer to restriction enzyme digests of yeast DNA. The lower band in the *Eco*RI digest is at 1.75 Md and is due to hybridization with the *CYC1* gene.

The efficiency of the hybridization declined rapidly above 45°, and at lower temperatures many additional bands were seen. Only one minor band remained at 44°.

We have used the labeled 15-mer to screen a λ phage bank of *Eco*R1 fragments of total yeast DNA. With the conditions optimized as described above, and using the plaque transfer procedure,[12] we observed a clear pattern of hybridization with individual plaques; however, the sensitivity was greatly enhanced by spotting the plaques to a grid before proceeding with the plaque transfer. In addition to the regular pattern, plaques prepared in this manner gave stronger signals and were larger in size, making them more distinct from the background (Fig. 4). Analysis of these clones has shown both the *CYC1* gene and the cloned fragment responsible for the minor band seen in Fig. 3.

Hybridization of a synthetic 13-mer with restriction enzyme digests of total yeast DNA has been reported by Montgomery *et al.*[17] They found hybridization with seven different fragments, including the *CYC1* gene. The correct band was identified with the *Eco*R1 site mutant described above. A *CYC1* clone was also identified by hybridization with a 13-mer. DNA of the correct size was isolated from a gel and used to prepare a phage λ bank. The plaques were transferred to a grid and screened by hybridization with labeled 13-mer. Hybridization with a clone carrying the *CYC1* gene was detected.

[17] D. L. Montgomery, B. D. Hall, S. Gillam, and M. Smith, *Cell* 14, 673 (1978).

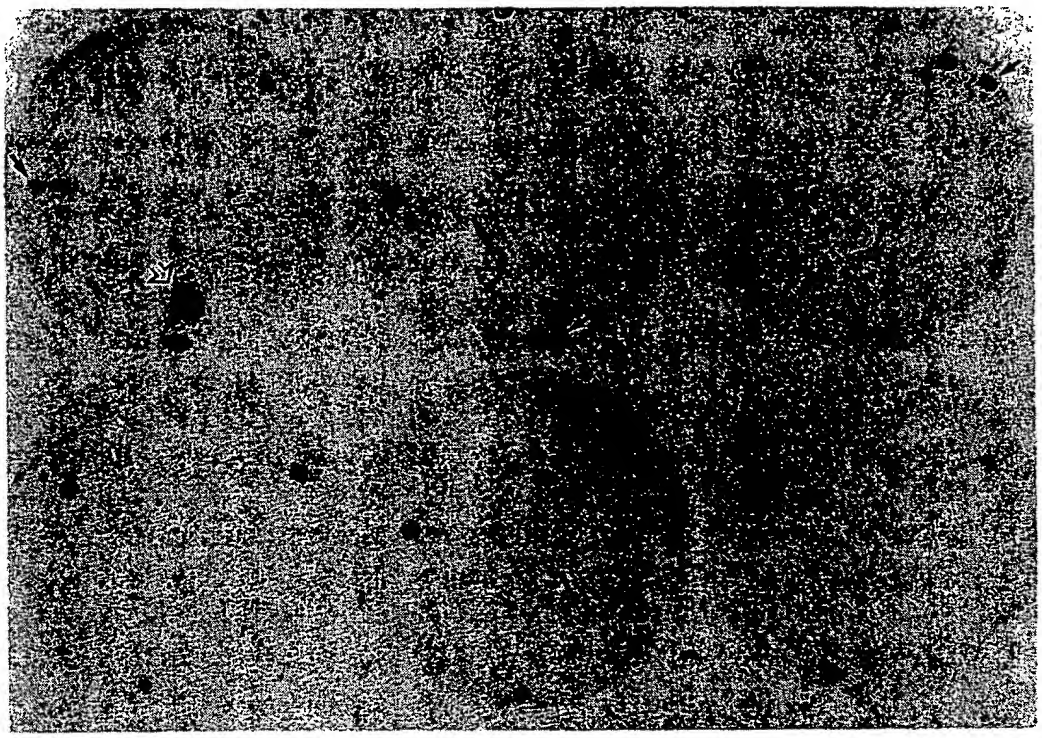

FIG. 4. Hybridization of labeled 15-mer with a λ phage bank of *Eco*RI fragments of total yeast DNA. Plaques transferred to a grid so that there were approximately 200 plaques per plate. The three common spots on all filters are a previously isolated clone containing the minor band seen in the *Eco*RI track in Fig. 3. The solid arrows indicate positive plaques and the open arrow nonspecific binding.

Problems with Filter Hybridizations

A common technical problem encountered in filter hybridization is a heavy background of dark spots on the film, which can obscure the desired bands. This problem becomes severe with the very high levels of radioactive probe used for oligonucleotide hybridization. The background can be minimized by using the lowest possible concentration of probe (usually 10^5–10^6 cpm/ml is sufficient) and by performing the hybridization in 0.2% polyvinylpyrrolidone, Ficoll, and bovine serum albumin. It is also important that the filter be thoroughly wetted before the labeled probe is added. Nitrocellulose filters wet poorly in high salt; the best procedure is to wet the filters in distilled water. They are then sealed in plastic bags and incubated briefly in hybridization buffer at 65°, after which the labeled probe is added (R. Rothstein, personal communication).

Cloning Eukaryotic Genes

The yeast genome contains 2×10^7 base pairs of DNA. A sequence of 12 nucleotides is likely to be unique. Since neither a 13-mer (seven bands)

nor a 15-mer (two bands) hybridized with a single site, the hybridization conditions were probably not sufficiently stringent to eliminate binding to closely related sequences. The complexity of mammalian DNA requires a 15- to 16-nucleotide-long sequence to be unique, and probably an 18- to 20-nucleotide sequence is more desirable to avoid unwanted hybridization with related sequences. This would be difficult to derive from the amino acid sequence of a protein. An alternative may be to screen a phage bank with two shorter probes 11–14 nucleotides in length and to search for clones to which both short probes bind.

Recently the phage M13 has been modified so as to be a useful cloning vector.[18] This phage produces plaques containing up to 10^9 phage particles. Plaque hybridization with this system is approximately 100 times more efficient than with λ plaques (R. Rothstein, personal communication). This property may greatly enhance the usefulness of synthetic oligonucleotides in screening phage banks. Probes short enough to be derived from amino acid sequences may be sufficiently long to have the sensitivity required for M13 plaque hybridization.

Acknowledgments

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[18] B. Gronenborg and J. Messing, *Nature (London)* **272**, 375 (1978).

[30] *In Situ* Immunoassays for Translation Products

By DAVID ANDERSON, LUCILLE SHAPIRO, and A. M. SKALKA

One approach to the isolation of specific recombinants from a clone bank has been immunological detection of cloned gene translation products produced in *Escherichia coli*. Immunological selection provides an approach when it is not possible to use selection techniques that depend

[1] A. Skalka and L. Shapiro, *Gene* **1**, 65 (1976).
[2] B. Sanzey, O. Mercereau, T. Ternynck, and P. Kourilsky, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3394 (1976).

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on nucleic acid hybridization or functional expression of the cloned gene. The first procedures developed for immunological selection were *in situ* immunoassays.[1,2] Recombinant phage or *E. coli* containing recombinant plasmids are grown on agar plates containing antisera produced against the protein encoded in the desired gene. Phage or *E. coli* expressing antigenic determinants of the protein are detected by observation of an immunoprecipitin reaction or an amplification of the immunoprecipitin reactions in the agar. Recently, other procedures have been developed that utilize radioiodinated antibody or radioiodinated protein A from *Staphylococcus aureus* and have the theoretical ability to detect one antigen molecule per *E. coli* cell.[3,4] However, the value of the *in situ* immunoassay remains, because of its simplicity. It is not necessary to iodinate protein or purify antibody from antisera or $F(ab)_2$ fragments of antibody. Furthermore, the *in situ* immunoassay itself is quite sensitive. Using *E. coli* β-galactosidase as a model antigen, it can be detected at the uninduced level which is in the range of 10–20 molecules per cell.[5]

Materials and Reagents

Reagents

1. N-Z amine A: Humko Sheffield Chemical, Norwich, New York
2. Salt free lysozyme: Worthington Biochemical Corporation, Freehold, New Jersey
3. Agarose: Seakem powder, Marine Colloids, P. O. Box 308, Rockland, Maine, or Sigma Chemical Company, Saint Louis, Missouri
4. Bacto-agar, Bacto-tryptone, and casamino acids: Difco Laboratories, Detroit, Michigan
5. 5-Bromo-4-chloro-3-indolyl-β,D-galactoside (XG): Bachem, Marina Del Ray, California
6. Isopropyl-β-thiogalactoside (IPTG): Sigma Chemical Company, Saint Louis, Missouri
7. Sarkosyl NL97: Ciba-Geigy Corporation, Greensboro, North Carolina
8. Rabbit anti-β-galactosidase serum: Gift of Dr. A. Fowler, University of California, Los Angeles

[3] H. Erlich, S. Cohen, and H. McDevitt, *Cell* 13, 681 (1978).
[4] S. Broome and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* 75, 2764 (1978).
[5] J. Miller, "Experiments in Molecular Genetics," p. 398. Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1972.